

Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/102248/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Zhang, Rongmin, Liu, Zhihai, Li, Jiyun, Lei, Lei, Yin, Wenjuan, Li, Mei, Wu, Congming, Walsh, Timothy Rutland ORCID: <https://orcid.org/0000-0003-4315-4096>, Wang, Yang, Wang, Shaolin and Wu, Yongning 2017. Presence of VIM-positive pseudomonas species in chickens and their surrounding environment. Antimicrobial Agents and Chemotherapy 61 (7) , e00167-17. 10.1128/AAC.00167-17 file

Publishers page: <http://dx.doi.org/10.1128/AAC.00167-17>
<<http://dx.doi.org/10.1128/AAC.00167-17>>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies.

See

<http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



Key words: chicken, fly, swallow, carbapenem resistance, *Pseudomonas* spp.

26 Metallo- β -lactamase gene *bla*_{VIM} was identified on the chromosome of four
27 *Pseudomonas* spp. isolates from a chicken farm, including one *P. aeruginosa* from
28 swallow (*Yanornis martini*), one *P. putida* from fly, and two *P. putida* from
29 chickens. The four isolates shared two variants of *bla*_{VIM}-carrying genomic
30 contexts, which resemble the corresponding regions of clinical MBL-producing
31 *Pseudomonas* species. Our study suggests the surveillance of
32 carbapenemase-producing bacteria in livestock and their surrounding
33 environment is urgently needed.

34

35 Carbapenems are critically important antimicrobials as a last line of defense against
36 multidrug-resistant Gram-negative bacterial infections (1, 2). As such, the increasing
37 prevalence of carbapenemase-producing isolates in the animal husbandry is of great
38 concern. While the metallo- β -lactamase (MBL) producing bacteria has been
39 commonly identified from food animals (3-7), *bla*_{MBL}-carrying *Pseudomonas* spp. are
40 very rarely reported in the animal husbandry or the surrounding environment.
41 Although we have reported the high prevalence of NDM in Enterobacteriaceae from
42 poultry production of Shandong province (7), carbapenemase-producing
43 non-Enterobacteriaceae isolates has not been identified from the same region. Here,
44 we report four chromosome-borne VIM-positive *Pseudomonas* isolates: one *P.*
45 *aeruginosa* isolates from a swallow (*Yanornis martini*), one *P. putida* isolate from a
46 fly, and two *P. putida* isolates from chickens. The *bla*_{VIM-2} was identified in the *P.*
47 *aeruginosa* isolate, but 27-bp at the 3'-terminal region of *bla*_{VIM-2} was truncated by an

48 IS6100 element in three *P. putida* isolates, resulting in a new variant of *bla*_{VIM} gene,
49 which was designed *bla*_{VIM-48}, and the *bla*_{VIM}-carrying genomic regions in these
50 *Pseudomonas* spp. isolates closely resembled the corresponding regions of clinical
51 MBL-producing *Pseudomonas* species.

52 Ninety-eight non-duplicated samples were randomly collected with an informed
53 consent form from a commercial chicken farm in Shandong Province, China (chicken
54 cloaca swabs, n=30; flies, n=30; dog anal swabs, n=17; swallow fecal swabs, n=10;
55 farmer fecal swabs, n=6; sewage, n=5), the procedures for the collection of all
56 samples were consistent with previous report (7). All samples were plated on
57 CHROMagar *Pseudomonas* (CHROMagar™ Paris, France) containing 8 µg/ml
58 meropenem (Ouhe Technology Company, Beijing, China). Putative *Pseudomonas*
59 colonies with blue color were recovered from 17 samples, and one colony from each
60 sample were identified to the species level by 16S rRNA sequencing (8). Of these,
61 four isolates were *bla*_{VIM} positive confirmed by PCR and sequencing (9). Further
62 MALDI-TOF MS (BrukerDaltonik GmbH, Bremen, Germany) analysis confirmed the
63 four positive isolates as a *P. aeruginosa* isolate from swallow fecal swabs, including
64 DZ-B1, a *P. putida* isolate from a fly, DZ-F23, and two *P. putida* isolates, DZ-C20
65 and DZ-C18, from chicken cloaca swabs.

66 MIC analysis (10, 11) showed that the four *Pseudomonas* spp. isolates were
67 almost resistant to all β-lactam antibiotics tested, including meropenem, imipenem,
68 aztreonam, and ceftazidime, with only isolate DZ-B1 showing susceptibility to
69 aztreonam (4 µg/ml) (Table 1). To further investigate the genetics background of four

70 isolates, whole genome sequencing was conducted according to previous report (7),
71 and draft assembly sequences were searched against the antibiotic resistance gene
72 database (<https://cge.cbs.dtu.dk/services/ResFinder/>), which confirmed the presence
73 of *bla*_{VIM} and other antibiotic resistance genes (Table 1). Additionally, in order to
74 investigate the location of the resistance element, the genomics DNA of four
75 VIM-producing isolates were digested with I-CeuI and separated by PFGE. The result
76 and Southern blot analysis showed that *bla*_{VIM} was located on the chromosome of all
77 four *Pseudomonas* isolates (Fig. S1). Moreover, the core genome phylogenetic
78 analysis was conducted to reveal the relationship between four isolates and other
79 known *Pseudomonas* isolates carrying *bla*_{VIM} from NCBI database (Table S1) using
80 Parsnp program (12). The phylogenetic tree revealed three *bla*_{VIM}-carrying *P. putida*
81 isolates showed distinct genomic heterogeneity, which is consistent with PFGE
82 analysis in SpeI patterns (Fig. S1). The ST385 of *P. aeruginosa* isolate DZ-B1 from
83 our study is confirmed by WGS. This ST type has previously been associated with
84 clinical *P. aeruginosa* isolates from India (13). The genome context of DZ-B1 were
85 closely related to that of seven *bla*_{VIM-2} carrying clinical *P. aeruginosa* from
86 Genebank (Fig. S2).

87 Four *bla*_{VIM}-carrying contigs, were identified from four isolates and confirmed by
88 PCR and sequencing, primers listed in Fig. S3. Analysis of the flanking regions of
89 *bla*_{VIM-2} on the chromosome of DZ-B1 revealed that it was located in a Tn5090-like
90 transposon bracketed by two 25-bp inverted repeats, IRI and IRT (Fig. A), suggesting
91 that the whole region could be mobilized using the *tni* machinery (15, 16). This

complete transposon shared 92.3% (7296/7907) nucleotide sequence identity to a *P. aeruginosa* Tn5090-like transposon, also containing *bla*_{VIM-2} gene, isolated from a Chinese patient (accession no. AM993098.1), and even greater sequence identity (99.4%, 4147/4171) was found within the corresponding *tniC-bla*_{VIM-2}-*aacA4-dhfr2* gene array located at the 3' end of the Tn5090-like transposon. The Tn5090-like transposon contained a conserved segment with an integrase gene, *intI1*, at the 3' end. *IntI1* is associated with the integration of resistance gene cassette array *bla*_{VIM-2}-*aacA4-dhfr2*, which is in the opposite orientation and confers resistance to carbapenems, some aminoglycosides, and trimethoprim, respectively. Another conserved gene cluster, *tniA-tniB-hp-tniC*, was located at the 5' end of the transposon (15). *TniA* and *tniB* were predicted to be involved in the transposition, while *tniC* codes for a recombination protein that differs from those encoded by *intI1*, *tniA*, and *tniB* (Fig. A) (15). This gene arrangement, in combination with *intI1*, involves in the transfer of a *bla*_{VIM-2}-carrying cassette in clinical *Pseudomonas* species (17-20).

A 9,875-bp fragment containing the *bla*_{VIM-2} like gene was observed in three *P. putida* strains, which sharing 99.9% sequence identity. Comparison analysis revealed that the *bla*_{VIM-2} like gene had a 27-bp deletion at the 3' terminal region, including its original stop codon, compared with the 801-bp *bla*_{VIM-2} in both DZ-B1 and the previously reported *P. aeruginosa* isolates. Immediately downstream of the 27-bp deletion was an intact insertion element, IS6100, and the truncated *bla*_{VIM-2} gene appeared to be terminated at the TAG stop codon located in the right inverted repeat of IS6100. This resulted in an 810-bp ORF, designed VIM-48 (KY362199), that was

114 9-bp longer than the previously reported gene (Fig. B). Further analysis of the
115 flanking regions revealed that it was very similar to the Tn6217 region in
116 *bla*_{IMP-9}-carrying plasmid pOZ176 from a clinical *P. aeruginosa* isolate from
117 Guangzhou, China (21). The upstream and downstream regions of *bla*_{VIM-48} contained
118 the *aacA4-int11*-Tn1403-like and *sul1-hp-IS6100* gene clusters, respectively. These
119 two regions shared 99.9% (2792/2794) and 99.9% (5726/5733) nucleotide sequence
120 identity, respectively, to the corresponding regions of Tn6217; however, the
121 downstream fragment was inverted, perhaps leading to the missing of 27-bp in the
122 3'-end of *bla*_{VIM-2} (Fig. A).

123 Similar to the Tn5090-like transposon in *P. aeruginosa* isolate DZ-B1, a 3,801-bp
124 segment harboring the *IS6100-bla*_{VIM-48}-*aacA4-int11* gene cluster in the three *P. putida*
125 isolates was bracketed by two 25-bp inverted repeats, IRi and IRt, suggesting that it
126 reached its current location by transposition (Fig. A) (22). Moreover, compared with a
127 6,942-bp fragment in the 3' region of the *bla*_{VIM-2}-carrying segment in *P. aeruginosa*
128 DZ-B1, only a 405-bp segment containing *dhfr2* was absent from the *P. putida* strains.
129 The remaining two nucleotide fragments were highly similar, sharing 100% (1
130 444/1444) and 99.9% (5091/5097) identity, respectively (Fig. A).

131 The 810-bp *bla*_{VIM-48} gene were amplified by PCR and confirmed by sequencing
132 using primers listed in Fig. S3, and then cloned into vector pHSG398 (Takara, Dalian,
133 China), which resulting a recombinant plasmid pHSG398-V01. Both the vector and
134 recombinant plasmid were introduced into *E. coli* Dh5 α (Takara, Janan) by
135 electropotation, respectively, resulting in two recombinant *E. coli* strains, designed

136 HSG398-V01 and HSG398-Dh5 α . MIC analysis showed that HSG398-V01 was more
137 active against imipenem and ceftazidime with 4 and 8 fold increase, respectively,
138 when comparing with HSG398-Dh5 α (Table 1), which suggested this *bla*_{VIM-48} gene is
139 functional in the three *P. putida* isolates. Phenotypic detection of
140 metallo- β -lactamases were performed on the three *P. putida* according to the previous
141 report (23), and synergy between imipenem and EDTA was observed for all three
142 strains (data not shown).

143 To date, 47 variants of *bla*_{VIM} gene have been reported (24), and all of them were
144 801-bp length and differ in several amino acids, while the *bla*_{VIM48} gene in this study
145 was 810-bp length, possibly due to the inverted insertion of IS6100-containing
146 fragment, revealing a new generation mechanism for the variant of *bla*_{VIM}
147 gene. Similar to the spread of *bla*_{VIM-2} in bacteria of clinical origin, the mobile genetic
148 elements, such as Tn5090, Tn1403, *int11* and IS6100 may play an important role in
149 the dissemination of the *bla*_{VIM} gene and its conserved flanking regions in bacteria
150 among food animals (17, 18, 25). Our study has found not only MBL-producing
151 *Pseudomonas* isolates in the livestock, but also in their surrounding environment,
152 suggesting the surveillance of carbapenemase-producing bacteria in livestock and
153 their surrounding environment is urgently needed.

154

155 **Nucleotide sequence accession numbers.**

156 All genome assemblies of 4 strains were deposited in GenBank and are registered
157 under BioProject accession number PRJNA381373, and the new VIM enzyme was
158 designed VIM-48 and deposited in GenBank under accession number KY362199.

159

160 **Acknowledgements**

161 This work was supported in part by National Natural Science Foundation of China
162 (Nos. 31572568, 31422055 and 81661138002), the National Key Basic Research
163 Program of China (No. 2013CB127200). T.R.W. was also supported by MRC grant
164 DETER-XDR-CHINA (MR/P007295/1).

165

166 **References**

- 167 1. **Nordmann P, Gniadkowski M, Giske CG, Poirel L, Woodford N,**
168 **Miriagou V, European Network on C.** 2012. Identification and screening of
169 carbapenemase-producing Enterobacteriaceae. Clin Microbiol Infect
170 **18:432-438.**
- 171 2. **Zhanel GG, Wiebe R, Dilay L, Thomson K, Rubinstein E, Hoban DJ,**
172 **Noreddin AM, Karlowsky JA.** 2007. Comparative review of the
173 carbapenems. Drugs **67:1027-1052.**
- 174 3. **Guerra B, Fischer J, Helmuth R.** 2014. An emerging public health problem:
175 acquired carbapenemase-producing microorganisms are present in
176 food-producing animals, their environment, companion animals and wild birds.
177 Vet Microbiol **171:290-297.**
- 178 4. **Zhang R, Wang Y, Liu Z, Li J, Yin W, Lei L, WU C, Shen J.** 2015.
179 Characterization of NDM-1-producing carbapenemase in *Acinetobacter* spp.
180 and *E. coli* isolates from diseased pigs. Frontiers of Agricultural Science and

- 181 Engineering 2:223.
- 182 5. **Woodford N, Wareham DW, Guerra B, Teale C.** 2014.
- 183 Carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae
- 184 from animals and the environment:an emerging public health risk of our own
- 185 making?. 2014. Journal of Antimicrobial Chemotherapy **69**:287-91.
- 186 6. **Wang Y, Wu C, Zhang Q, Qi J, Liu H, He T, Ma L, Lai J, Shen Z, Liu Y,**
- 187 **Shen J.** 2012. Identification of New Delhi metallo-beta-lactamase 1 in
- 188 *Acinetobacter lwoffii* of food animal origin. PLoS One **7**:e37152.
- 189 7. **Wang Y, Zhang R, Li J, Wu Z, Yin W, Schwarz S, M. Tyrrell J, Zheng Y,**
- 190 **Wang S, Shen Z, Liu Z, Liu J, Lei L, Li M, Zhang Q, Wu C, Zhang Q, Wu**
- 191 **Y, Walsh T, Shen J.** 2017. Comprehensive resistome analysis reveals the
- 192 prevalence of NDM and MCR-1 in Chinese poultry production. Nature
- 193 Microbiology. Doi: 10.1038/nmicrobiol.2016.260.
- 194 8. Kim T-W. 2010. Identification and distribution of Bacillus species in doenjang
- 195 by whole-cell protein patterns and 16S rRNA gene sequence analysis. Journal
- 196 of Microbiology and Biotechnology 20:1210-1214.
- 197 9. **Gutierrez O, Juan C, Cercenado E, Navarro F, Bouza E, Coll P, Perez JL,**
- 198 **Oliver A.** 2007. Molecular epidemiology and mechanisms of carbapenem
- 199 resistance in *Pseudomonas aeruginosa* isolates from Spanish hospitals.
- 200 Antimicrob Agents Chemother **51**:4329-4335.
- 201 10. **Clinical and Laboratory Standards Institute.** 2015. Performance standards
- 202 for antimicrobial susceptibility testing; twenty-fifth informational supplement.

- 203 CLSI document M100-S25. CLSI, Wayne, PA, USA.
- 204 11. **Clinical and Laboratory Standards Institute.** 2015. Performance standards
205 for antimicrobial disk and dilution susceptibility tests for bacteria isolated
206 from animals. 3rd ed. CLSI supplement VET01S. CLSI, Wayne, PA, USA.
- 207 12. **Treangen TJ, Ondov BD, Koren S, Phillippy AM.** 2014. The Harvest suite
208 for rapid core-genome alignment and visualization of thousands of
209 intraspecific microbial genomes. *Genome Biol* 15:524.
- 210 13. **Kos VN, Deraspe M, McLaughlin RE, Whiteaker JD, Roy PH, Alm RA,**
211 **Corbeil J, Gardner H.** 2015. The resistome of *Pseudomonas aeruginosa* in
212 relationship to phenotypic susceptibility. *Antimicrob Agents Chemother*
213 **59**:427-436.
- 214 14. Lee C, Mariani KJ. 2013. Characterization of the nucleoid-associated protein
215 YejK. *J Biol Chem* 288:31503-31516.15. **Radstrom P, Skold O,**
216 **Swedberg G, Flensburg J, Roy PH, Sundstrom L.** 1994. Transposon
217 Tn5090 of Plasmid R751, Which Carries an Integron, Is Related to Tn7, Mu,
218 and the Retroelements. *z***176**:3257-3268.16. **Juan C, Zamorano L, Mena A,**
219 **Alberti S, Perez JL, Oliver A.** 2010. Metallo-lactamase-producing
220 *Pseudomonas putida* as a reservoir of multidrug resistance elements that can
221 be transferred to successful *Pseudomonas aeruginosa* clones. *Journal of*
222 *Antimicrobial Chemotherapy* **65**:474-478.
- 223 17. **Wright LL, Turton JF, Hopkins KL, Livermore DM, Woodford N.** 2015.
224 Genetic environment of metallo-beta-lactamase genes in *Pseudomonas*

- 225 *aeruginosa* isolates from the UK. J Antimicrob Chemother **70**:3250-3258.
- 226 18. **Santos C, Caetano T, Ferreira S, Mendo S.** 2010. Tn5090-like class 1
227 integron carrying *bla*_{VIM-2} in a *Pseudomonas putida* strain from Portugal. Clin
228 Microbiol Infect **16**:1558-1561.
- 229 19. **Lolans K, Queenan AM, Bush K, Sahud A, Quinn JP.** 2005. First
230 nosocomial outbreak of *Pseudomonas aeruginosa* producing an
231 integron-borne metallo-beta-lactamase (VIM-2) in the United States.
232 Antimicrob Agents Chemother **49**:3538-3540.
- 233 20. **Lagatolla C, Edalucci E, Dolzani L, Riccio ML, De Luca F, Medessi E,**
234 **Rossolini GM, Tonin EA.** 2006. Molecular evolution of
235 metallo-beta-lactamase-producing *Pseudomonas aeruginosa* in a nosocomial
236 setting of high-level endemicity. J Clin Microbiol **44**:2348-2353.
- 237 21. **Xiong J, Alexander DC, Ma JH, Deraspe M, Low DE, Jamieson FB, Roy**
238 **PH.** 2013. Complete sequence of pOZ176, a 500-kilobase IncP-2 plasmid
239 encoding IMP-9-mediated carbapenem resistance, from outbreak isolate
240 *Pseudomonas aeruginosa* 96. Antimicrob Agents Chemother **57**:3775-3782.
- 241 22. **Partridge SR, Brown HJ, Stokes HW, Hall RM.** 2001. Transposons Tn1696
242 and Tn21 and their integrons In4 and In2 have independent origins.
243 Antimicrob Agents Chemother **45**:1263-1270.
- 244 23. **Robin F, Aggoune-Khinache N, Delmas J, Naim M, Bonnet R.** 2010. Novel
245 VIM metallo-beta-lactamase variant from clinical isolates of Enterobacteriaceae
246 from Algeria. Antimicrob Agents Chemother **54**:466-470.

- 247 24. **Zamorano L, Mulet X, Garau M, Gallegos C, Juan C, Perez JL, Oliver A.**
248 2016. VIM-47, a New Variant of the Autochthonous Metallo-beta-Lactamase
249 VIM-13 from the Balearic Islands in Spain. *Antimicrob Agents Chemother*
250 60:3251-3252.
- 251 25. **Papagiannitsis CC, Miriagou V, Giakkoupi P, Tzouveleakis LS, Vatopoulos**
252 **AC.** 2013. Characterization of pKP1780, a novel IncR plasmid from the
253 emerging *Klebsiella pneumoniae* ST147, encoding the VIM-1
254 metallo-beta-lactamase. *J Antimicrob Chemother* 68:2259-2262.
255

256 **Figure Legends**

257 Figure 1

258 A: Genetic context of *bla*_{VIM} in *Pseudomonas* species isolates from chickens, a fly,
259 and a swallow, and structural comparison with the corresponding genetic regions in
260 Tn5090-like and Tn6217 transposons. The arrows indicate the positions and directions
261 of transcription of the genes. Different genes are indicated by different types of
262 shading. B The nucleotide sequence of the region encompassing the 3' ends of *IS6100*
263 and *bla*_{VIM-48}. The coding regions of the two open reading frames are marked by gray
264 shading, and the right inverted repeat (IRR) of *IS6100* is underlined.

265

Table 1 Antimicrobial susceptibility profiles of *Pseudomonas putida* isolates DZ-C20, DZ-C18, and DZ-F23, and *Pseudomonas aeruginosa* DZ-B1, and recombinant *E. coli* strains HSG398-V01 and HSG398-DH5 α .

DZ-B1, and recombinant <i>E. coli</i> strains HSG398-V01 and HSG398-DH5α.											
Bacterial isolate	Resistance genes	MIC (mg/liter)									
		MEM	IMI	ATM	CAZ	GEN	CIP	COL	TGC	TMP	
DZ-C18	<i>bla</i> _{CARB-4'} <i>bla</i> _{VIM48'} , <i>strA</i> , <i>strB</i> , <i>aacA4</i> , <i>aadA13</i> , QnrVC1, <i>mph(A)</i> , <i>cmlA1</i> , <i>floR</i> , <i>tet(A)</i> , <i>tet(G)</i> , <i>sulI</i>	128	128	256	128	64	32	2	8	≥256	
DZ-C20	<i>bla</i> _{CARB-4'} <i>bla</i> _{VIM-48} , <i>strA</i> , <i>strB</i> , <i>aacA4</i> , <i>aphA6</i> , QnrVC1, <i>mph(A)</i> , <i>floR</i> , <i>catB</i> , <i>tet(G)</i> , <i>sulI</i>	64	32	128	128	128	32	4	2	≥256	
DZ-F23	<i>bla</i> _{CARB-4'} <i>bla</i> _{VIM-48'} <i>strA</i> , <i>strB</i> , <i>aacA4</i> , <i>aphA6</i> , QnrVC1, <i>mph(A)</i> , <i>floR</i> , <i>tet(G)</i> , <i>sulI</i>	64	32	32	32	16	32	1	8	≥256	
DZ-B1	<i>bla</i> _{VIM-2'} <i>bla</i> _{OXA-50'} <i>ampC</i> , <i>strA</i> , <i>strB</i> , <i>aacA4</i> , <i>aph(3')-IIa</i> , <i>aph(3')-IIb</i> , <i>aac(6')-IIa</i> , <i>catB7</i> , <i>floR</i> , <i>tet(G)</i> , <i>sulI</i> , <i>dfrB1</i> , <i>fosA</i>	32	32	4	64	256	256	2	8	≥256	
HSG398-V01	<i>bla</i> _{VIM-2} -like	0.125	1	0.032	2	-	-	-	-	-	
HSG398-DH5α	-	0.0625	0.25	0.032	0.125	-	-	-	-	-	

The antimicrobial agents are abbreviated as follows: MEM, meropenem; IMI, imipenem; ATM, aztreonam; CAZ, ceftazidime; GEN, gentamicin; CIP, ciprofloxacin; COL, colistin; TGC, tigecycline; TMP, trimethoprim.

Fig. A

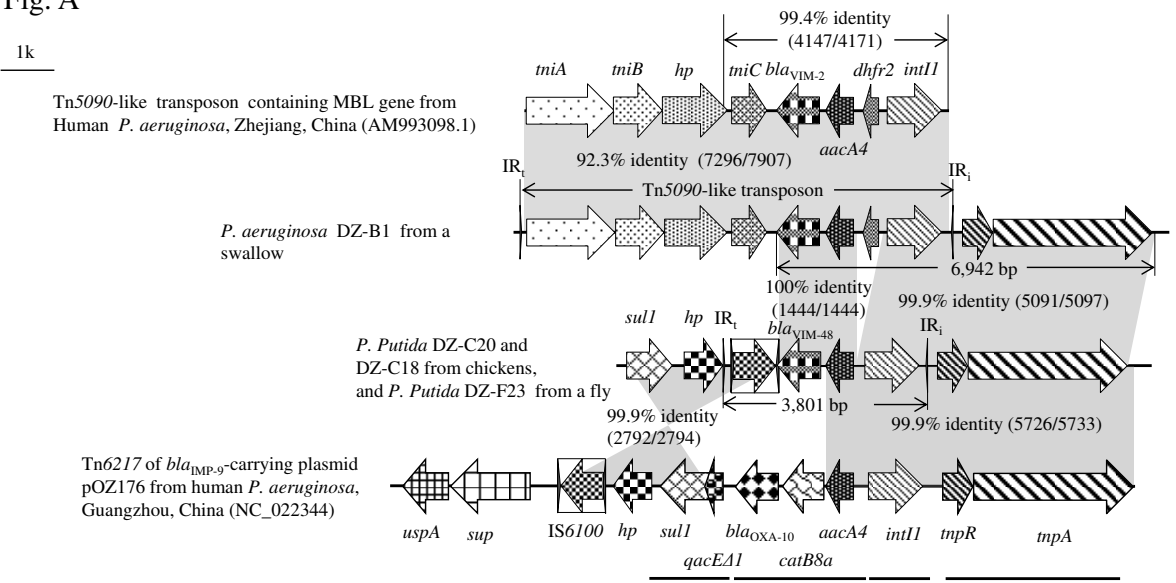


Fig. B

